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13. SUPPLEMENTARY NOTES

14. ABSTRACT
Epidemiological studies show that B, Se and Znreduce prostate cancer risk whereas Ca and Cd increase risk. The objective of this proposal was to determine the concentration and location of these elements in normal and tumor tissue. Specific aims included: (1) preparation of Gleason graded prostate tissue, (2) determination of tissue concentrations of: B, Ca, Cd, Se and Zn; and (3) determination of tissue and cellular distribution of these elements using a NanoSIMS ion microscope at Lawrence Livermore National Laboratory (LLNL). Specific aims 1, 2 were accomplished and showed that B was unique in its variability. Further study identified B as a chemopreventative agent and a clinical trial has been designed to determine if it lowers PSA level in men. Aim 3 was accomplished, but future studies are needed to improve the resolution of intracellular mapping of the elements. The work on B provided important insights on how the movement of calcium from the endoplasmic reticulum into the cytoplasm can be modulated by chemopreventative agents to reduce cell proliferation. This finding opens new opportunities for cancer prevention and control.

15. SUBJECT TERMS
Boron, selenium, zinc, calcium, cobalt, cancer prevention, risk factors

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Introduction

There is growing evidence that the elements boron, selenium and zinc reduce PCa risk whereas calcium and cadmium increase risk (1-12). The objective of this proposal was to determine if these elements differ in concentration and location between normal and tumor tissue and thus help to identify chemopreventative modes and mechanisms of action. The project was able to make substantial progress toward identifying boron as a new chemopreventative element with a new mode of chemoprevention. However, the tissue concentration data on selenium, zinc and cadmium did not substantiate significant associations between them and the severity of the disease.

Pathologically graded human prostate was analyzed to determine the elemental concentrations in these tissues and their relationship with Gleason scores. The results showed no significant differences in gross concentrations and Gleason scores (13). However, the variability of the element boron (B) was intriguing and so the chemopreventative properties of B were examined in human prostate cell lines. These studies showed B had strong chemopreventative properties and future studies should examine if it is efficacious in a clinical trial (14-20). Analyzing the intracellular location of the elements was difficult. The art and science of biological sample preparation for NanoSIMS analysis is primitive, but images were obtained that showed the elements were compartmentalized within cells. We are continuing to work with scientists at the Lawrence Livermore National Laboratory's NanoSIMS microscopy laboratory to improve the resolution. However, at the end of this grant the NanoSIMS procedure is still unable to provide unambiguous results on the intracellular localization of the elements in human tissue. On a positive side we were able to make significant progress in the characterization of boron as a chemopreventive agent. This included the identification of a physiological mode of action a potential molecular target that may explain its chemopreventative action.

Key Research Accomplishments

Task 1. To identify and maintain a series of progressively dedifferentiated samples.

Accomplishments Project year 1: Unmatched and matched sets of normal and tumor prostate tissue were collected from the UCLA Human Tissue Research Center. Unmatched samples were used to establish the methods for elemental analysis. We had to use normal tissue from men with cancer because samples of normal tissue from normal men are nearly none existent. The vast majority of tissue samples available have Gleason scores in the range from 6 to 7.

Task 2. To utilize state of the art inductive coupled plasma mass spectrometry to determine the concentration of the B,Ca, Cd, Se and Zn in whole tissue samples. .

Accomplishments Project year 2: We analyzed matched pairs of normal and tumor tissue to determine the concentrations of B, Ca, Cd, Se and Zn in 23 different male donors. The mean, median, range and coefficient of variation of the concentrations are given in Table 1.

Table 1. Elemental Concentrations of Normal and Tumor Prostate Tissue in 23 Men						
Normal		Boron	Cadmium	Calcium	Selenium	Zinc
		ng/g	ng/g	µg/g	ng/g	µg/g
	Mean	291	71	313	471	86
	Median	90	58	300	480	73
	Range	28 - 3530	8 - 220	82 - 830	200 - 730	9 - 190
	Std Error	159	10	36	24	11
	CV	256%	66%	56%	25%	63%
Tumor						
	Mean	355	83	263	502	88
	Median	100	61	270	520	84
	Range	23 - 2360	20 - 250	80 - 430	15 - 820	110-180
	Std Error	129	12	18	34	9
	CV	170%	70%	33%	33%	50%

The concentrations in both normal and tumor tissue ranked as follows: Ca > Zn > Se > B > Cd. A statistical comparison of elemental concentrations in normal and tumor tissue did not reveal significant differences (Table 2). The coefficient of variation of the elements varied greatly between elements (Table 1). The magnitude of variation followed the same rank in normal and tumor tissue: B > Cd > Zn > Ca > Se. The high variation in boron concentrations was not expected. Boron is not known to activate or covalently bind to proteins. It's variability and 10 fold range in concentration suggests that prostate is able to accumulate boron. Examination of tissue using the NanoSIMS ion probe will determine where this occurs for boron and the other elements.

Table 2. Statistical Evaluation of Elemental Concentrations in Matched Normal and Tumor Tissue	
Element	Statistical Comparison between Normal and Tumor Tissue
Boron[1]	$p = 0.31$
Calcium[1]	$p = 0.46$
Cadmium[1]	$p = 0.58$
Selenium[1]	$p = 0.21$
Zinc[2]	$p < 0.09$

We then determined the strength of the relationship between whole tissue elemental concentrations and pathological tissue classification will be determined by statistical analysis.

Accomplishments: Table 3 shows there was no relationship between Gleason score and the concentration of any element.

Table 3. Statistical Evaluation of the Relationship between Gleason Scores and Elemental Concentrations in Tumor Tissue	
Element	Correlation Coefficient of Gleason Score versus Element Concentration
Boron[1]	R = 0.10
Calcium[1]	R = 0.13
Cadmium[1]	R = 0.08
Selenium[1]	R = 0.30
Zinc[2]	R = 0.21

Each of these elements had been positively or negatively associated with prostate cancer risk, but this did not show up as concentration differences at the gross tissue level. We were concerned that the variability may have come from dye contamination used in the Clinical Pathology Laboratory to mark regions of the organ. Dyes are applied to the outside of the prostate gland by clinical pathology laboratories to mark regions for technicians that subsequently prepare the gland for evaluation and Gleason grading. We analyzed the elemental composition of the three dyes used at UCLA. The results of triplicate measurements is given in the table below and show that element concentrations were too low to be a significant variable.

Table 4. Concentrations of elements in Pathology Laboratory Dyes					
Clinical Pathology Marker Dye	Boron ng/g	Cadmium µg/g	Calcium µg/g	Selenium µg/g	Zinc µg/g
Black	ND; ND; ND	ND; ND; 4	ND; 17; 16	ND; ND; ND	ND; ND; ND
Blue	ND; ND; ND	0.37; 0.39; 0.4	41; 39; 40	0.42; 0.34; 0.37	1.6; 1.5; 1.6
Yellow	ND; ND; ND	ND; ND; ND	ND; ND; ND	ND; ND; ND	ND; ND; ND
Limit of Detection	60	0.002	10	0.2	0.3
LD = limit of detection; ND = not detectable					

The source of the variability is most likely due to natural biological variability.

Task 3. To determine the microlocation and microconcentrations of B, Ca, Cd, Se and Zn in graded series of samples.

Accomplishments (project years 2 and 3): The NanoSIMS ion microscope was installed at Lawrence Livermore in the Fall of 2004 and the two scientists who ran it worked with us to obtain images of the elements in prostate cells. Ion imaging of

elements within prostate cells was accomplished using the ion microscope (NanoSIMS 50) located in the Analytical and Nuclear Chemistry Division of the Lawrence Livermore National Laboratory.

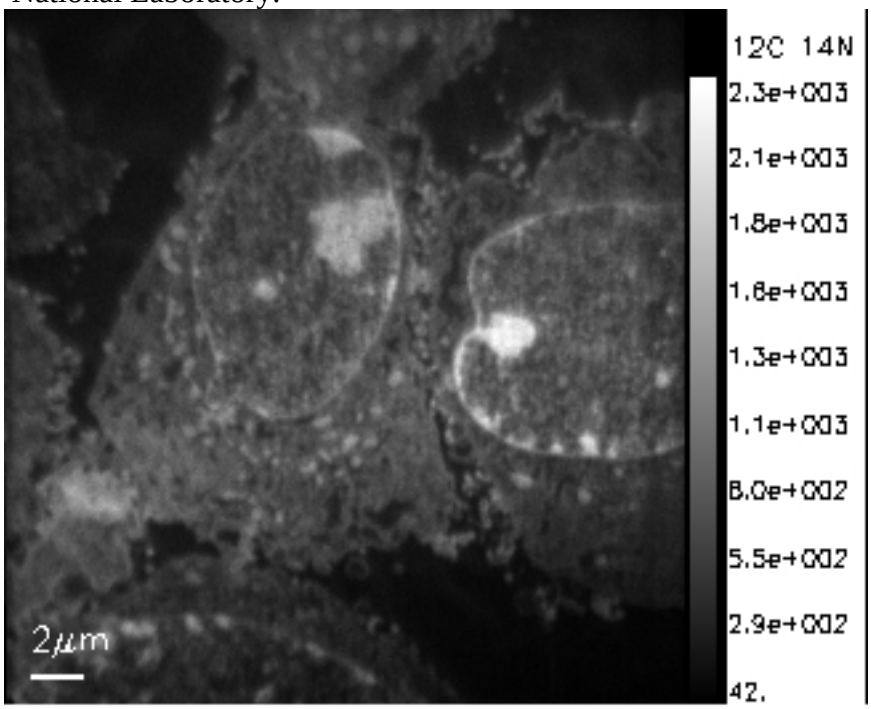


Figure 1. The light areas represent the origin of secondary carbon-nitrogen polyatomic ions sputtered from the sample by primary cesium ions rostered across the cells. The C-N atoms, which represent protein, are at their highest levels around the nucleus and within the nucleolus. The numbers to the right of the image indicate C-N polyatoms /per C emitted from the same region.

Problems:

Progress on Task 3 focused on the development of methods to prepare tissue for ion microscopy. We purchased a Leica EM MM80 slam freezer to ultra-rapidly freeze prostate tissue and a Thermo Neslab CC-100 cold probe to regulate the dehydration phase. Briefly, the Leica slam freezer was prechilled by filling with liquid nitrogen. The tissue was slam frozen and transferred to a Dewar containing acetone chilled to – 83^{o}C. The temperature of the acetone bath was controlled using the Thermo Neslab CC-100 probe and refrigeration unit. Samples were slowly brought to room temperature over a period of 72 hours. The dehydrated tissue was fixed in osmium and embedded in Spurr Low Viscosity Resin. This procedure was been successful using cultured prostate cells as the preparations look good when evaluated by scanning electron microscopy, however the acetone caused leakage of the membranes so techniques needed to be developed to fix and dehydrate cells and tissues without the use of membrane damaging agents.

Ice crystals form in tissues frozen at -80^{o}C or above. When tissues are dropped into liquid nitrogen, the temperature conductance through the tissue is inefficient and crystals

form before the tissue drops below -80^{o}C. We employed slam freezing to prevent this. Ice crystals also form if water is not removed before the tissue is defrosted. We used a freeze substitution method that replaced water with acetone. This worked, but we were concerned that it may also disrupt ion distribution in the tissue. In the third year of the project we switched to a Turbo Freeze Drier that holds samples down to -140^{o}C under vacuum and during the warm-up phase when samples cross the -80^{0}C barrier a second time. We are working with Scientist at Lawrence Livermore to determine if the resolution of images is greatly improved with our improved procedures and using prostate as a model system to develop the procedure for other human tissues. This grant has ended, but the work will be continued pending success in applications for funds from other granting agencies.

Accomplishments (project year 3 and no-cost time extension)

During year 3 and the no-cost time extension considerable progress was made toward understanding how boron mediates its preventative effects. We were able to identify a mode of action that may explain its ability to inhibit cell proliferation (figure 2-6). Boron modulated the release of intracellular calcium stores at human physiological concentrations by inhibiting the NAD/cADPR calcium release pathway. This is important, as the release of calcium stores activates numerous calcium binding proteins that regulate cellular processes, including the cell cycle. These observations provide the stage to identify potential boron molecular targets that are involved in modulating stored calcium release.

Studying the chemopreventive properties of boron has presented a difficult challenge. Boron does not have a stable radioactive isotope and physiological concentrations are near detectable limits the inductively coupled plasma mass spectrometry (ICPMS). All experiments require the use of ultrapure water, reagents and boron free labware. In addition students had to be trained to conduct all experiments using ultraclean procedures to avoid contamination.

The following figures show in order: binding of boric acid, the physiological form of boron, to NAADP and cADPR, these are both intracellular calcium release signaling molecules (fig 2); inhibition of NAD stimulated calcium release from the endoplasmic reticulum (fig 3); stereospecificity of boric acid's effect on inhibition of calcium release shown by the ineffectiveness of methylboronic acid (fig 3); stereospecificity of boric acid's effect on inhibition of prostate cancer cell proliferation as shown by the ineffectiveness of methylboronic acid (fig 4); stereospecificity of boric acid's effect on the redistribution of intracellular calcium stores in the DU-145 cell line and the ineffectiveness of methylboronic acid (fig 5)

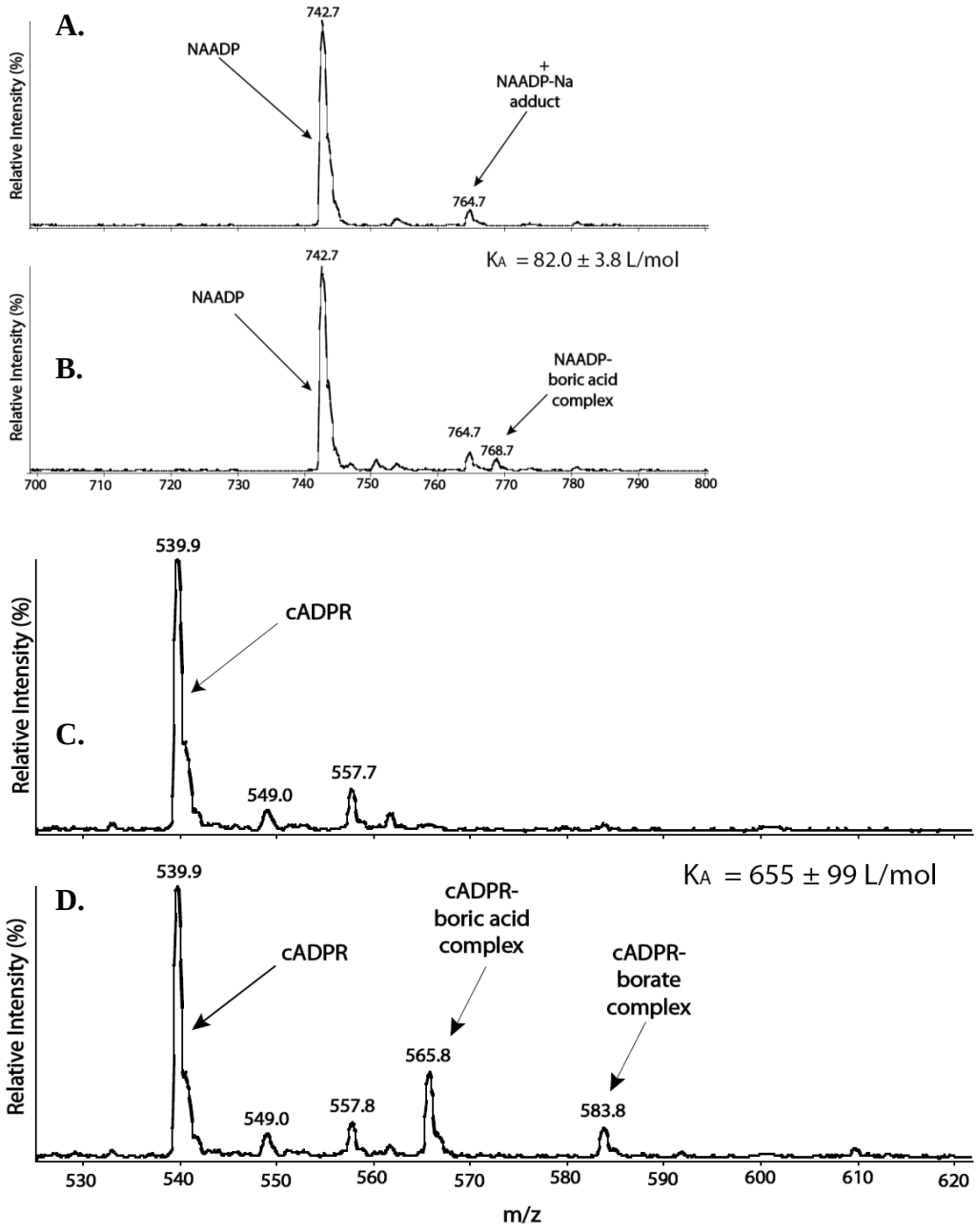
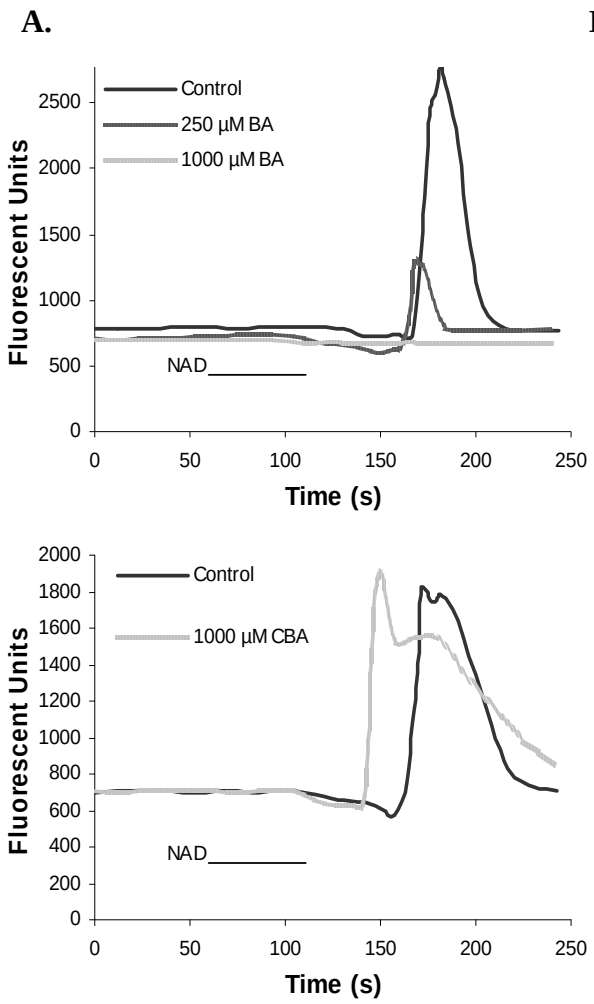

A.
742.7
NAADP
+
NAADP-Na
adduct
764.7
Relative Intensity (%)
KA = 82.0 ± 3.8 L/mol
B.
742.7
NAADP
NAADP-
boric acid
complex
764.7
768.7
Relative Intensity (%)
700
710
720
730
740
750
760
770
780
790
800
C.
539.9
cADPR
549.0
557.7
Relative Intensity (%)
KA = 655 ± 99 L/mol
D.
539.9
cADPR
cADPR-
boric acid
complex
cADPR-
borate
complex
565.8
549.0
557.8
583.8
Relative Intensity (%)
530
540
550
560
570
580
590
600
610
620
m/z

Figure 2. Negative ion ESI-MS spectra of NAADP, NAADP-BA complexes, cADPR and cADPR-BA complexes in water:acetonitrile:triethylamine mixtures at pH 10.3. (A) 100 µM NAADP showing an intense $(M-H)^-$ signal at m/z 742.7 (calcd 743.1 Da). The signal at m/z 764.7 (calcd 765.1) is assigned as NAADP-Na^+ adduct. (B) A mixture of 100 µM NAADP and 500 µM $^{11}B(OH)_3$ produced signals corresponding to the NAADP-BA complex at m/z 768.7 (calcd 769.1 Da). K_A for the complex formation was calculated as 82 ± 3.8 L/mol. (C) 100 µM cADPR showing an intense $(M-H)^-$ signal at m/z 539.9 (calcd 540.0 Da). The signals at m/z 549.0 and 557.8 are assigned as unknown impurities in the cADPR sample. (D) A mixture of 100 µM cADPR and 500 µM $^{11}B(OH)_3$ produced signals corresponding to the cADPR-BA complex at m/z 585.7 (calcd 586.0 Da), and the cADPR-borate complex at m/z 583.7 (calcd 584.0). K_A for the complex formation was calculated as 655 ± 99 L/mol.



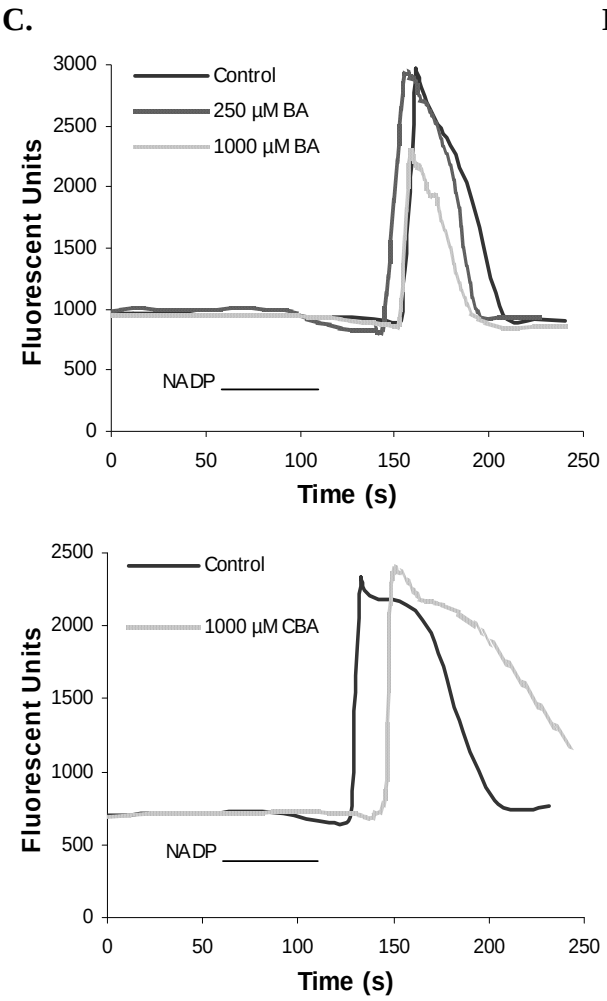


Figure 3. NAD^+ and $NADP^+$-induced Ca^{2+} release sensitivity in DU-145 cells treated for 8 days with BA and CBA (250 and 1000 µM). (A) BA treatment (250 and 1000 µM) inhibited NAD^+ (10 mM) induced Ca^{2+} release, whereas (B) CBA (1000 µM) treatment did not. (C) BA (1000 µM) treatment reduced $NADP^+$ (5 mM) induced Ca^{2+} release whereas (D) CBA (1000 µM) did not. Traces represent mean fluorescence (Fluo-4) of four individual cell responses.

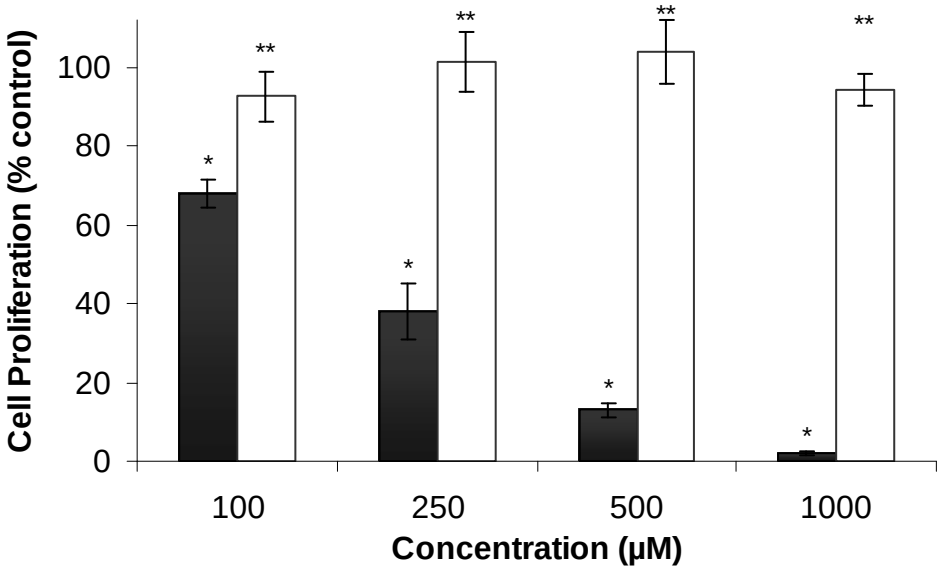
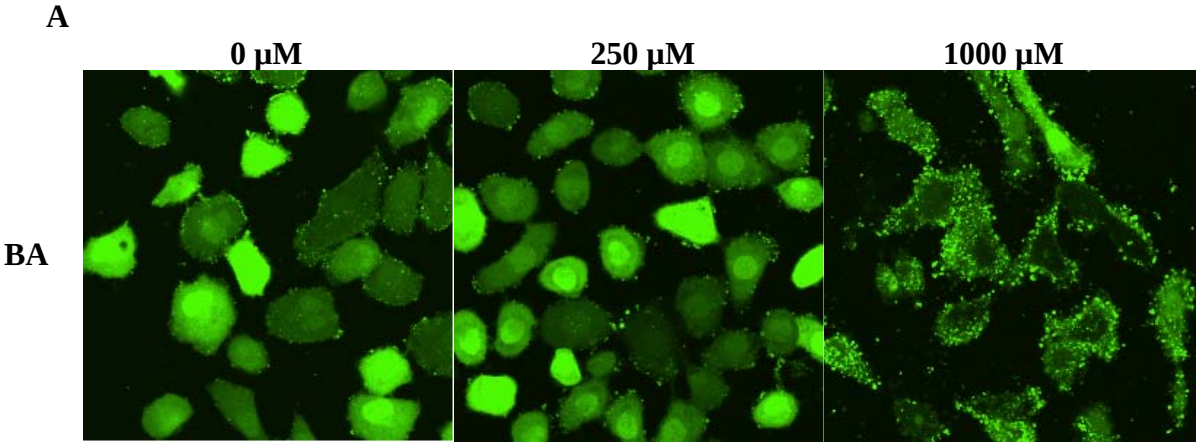


Figure 4. Effects of BA and CBA on DU-145 cell proliferation. A dose-dependent reduction in cell proliferation occurs in cells exposed to BA (■) for eight days (100-1000 μM), but not CBA (□). Values are presented as the mean of six independent measurements ± sem. (*) represents statistically significant means compared to the 0 μM BA control; (**) represents significant differences between BA and CBA at like dose.



CBA

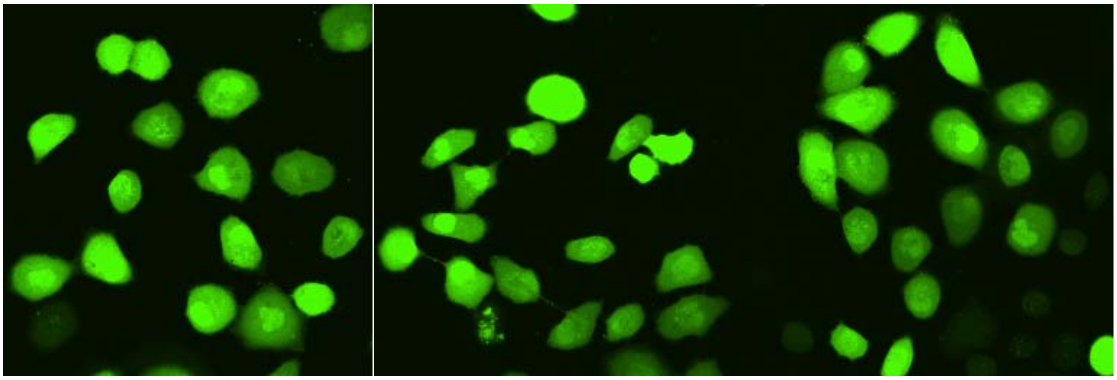


Figure 5. Intracellular Ca^{2+} distribution and concentration in DU-145 cells exposed to 24-hour and 8-day BA and CBA. (A.) Fluorescent images depicting intracellular Ca^{2+} re-distribution, from diffuse to sub-cellular localized, in DU-145 cells loaded with Fluo-4, following 8-day exposure to 0-1000 µM BA and CBA.

I also note that we received the following letter concerning our funds from this grant and so the no-cost time extension did not extend an entire year.

Ms. Conn,

Please reference subject grant under the direction of Dr. Curtis Eckhert.
We have received your SF 272 for the period 7-1-06 - 9-30-06.
I am very concerned that you have indicated a negative balance. Our records indicate you have received all payments thus far totaling $417,082. There are no payments remaining under this research project.

Please check your records for this grant as you had requested a one year extension without additional funds on 30 March 2006. Please plan to have Dr. Eckhert complete the statement of work. If you have no remaining funds, please have Dr. Eckhert stop research and submit his final report. Again, no further funds will be forthcoming. Thank you for your attention to this matter.

Sacelia L. Heller
Sacelia L. Heller
Contract Specialist
301-619-7349
301-619-3166 FAX

KEY RESEARCH ACCOMPLISHMENTS

- Successful procurement of Gleason graded tissue classified as normal, gleason grades 3, 5, 6, 7, 8.
- Analysis of matched sets of normal and tumor tissue obtained from 23 different men for the elements, boron, calcium, cadmium, selenium and zinc.

- Statistical analysis shows there is no difference between normal and tumor concentrations of these elements.
- Development of methods for NanoSIMS analysis for localizing elements within prostate cells.
- First ion microscope images of prostate cells obtained using the NanoSIMS instrument at Lawrence Livermore National Laboratory
- Significant progress made toward improving the preparation of prostate cells for higher resolution of the subcellular location of elements.
- Observed a 10 fold range of boron concentration in prostate tissue suggested the element differed significantly between individual men and maybe related to the risk of prostate cancer.
- Evaluation of the chemopreventative properties of boron with the outcome showing it is a strong chemopreventative agent.
- Identification that the physiological form of boron, boric acid, inhibits calcium signaling and inhibits cell proliferation of prostate cancer cells
- Identification of cADPR, a intracellular calcium release signaling molecule, as a molecular target of boric acid

REPORTABLE OUTCOMES

Manuscripts and Abstracts

Barranco WT, Stella Jr SL, Kim DH and Eckhert CD. Boric acid inhibits the NAD/CD38/cADPR/Calcium Signaling Pathway. Under review

Henderson K and Eckhert CD. The Effect of Boron on the UPR in Prostate Cancer Cells is Biphasic, FASEB J, May, 2007

Barranco WT, Hudak PF and Eckhert CD. Erratum to: Evaluation of ecological and in vitro effects of boron on prostate cancer risk. *Cancer Causes Control* 18:71-77, 2007, Published Online: http://dx.doi.org/10.1007/s10552-007-0077-8, *Cancer Causes Control*, March, 2007.

Barranco WT, Hudak PF and Eckhert CD. Evaluation of ecological and in vitro effects of boron on prostate cancer risk. *Cancer Causes Control* 18:71-77, 2007.

Barranco WT, Hudak PF and Eckhert CD. Evaluation of ecological and in vitro effects of boron on prostate cancer risk. *Cancer Causes Control* 18:71-77, 2007.

Kim DH, Que Hee S, Norris A, Faull KF and Eckhert CD. Boric acid inhibits ADP-ribosyl cyclase non-competitively. *J. Chromatography A.* 1115:246-252, 2006.

Barranco WT and Eckhert CD. Cellular changes in boric acid-treated DU-145 prostate cancer cells. *Brit J. Cancer* 94:884-890, 2006.

Henderson K and Eckhert CD. Boric acid induces ER stress in DU-145 and LNCAP prostate cancer cell lines. *Soc. Tox*. 2006.

Barranco WT and Eckhert CD. Inhibition of DU-145 Prostate Cancer Cell Proliferation by Boron and Selenium is Additive. *FASEB J.* 2005.

Barranco W.T. and Eckhert C.D. Boric acid inhibits human prostate cancer cell proliferation. *Cancer Letters* 216:21-29, 2004.

Kim D. H. S., Faull K. F., Norris A. J., Eckhert C. D. Borate-nucleotide complex formation depends on charge and phosphorylation state. *J. Mass Spectrometry* 39:743-751, 2004.

Barranco, W.T. Eckhert, C.D. Boric acid acts as a cADPR / RyR antagonist during inhibition of human prostate cancer cell proliferation. *FASEB J.* 2004; 18:A351.2 (352.2).

Kim, D.H, Faull, K.F., Eckhert, C.D. Determination of borate complex with cyclic ADP-ribose (cADPR) by electrospray ionization mass spectrometry (ESI-MS) *FASEB J.* 2004; 18: A351.4 (351.4).

Eckhert, C.D. Concentration and variation of boron, selenium and elements associated with cancer risk in non-tumor human prostate tissue. *FASEB J.* 2004; 18:A351.3 (351.3).

Employment and Research Opportunities

Support was provided for research training of the following students:

Kim Henderson (currently a Ph.D. working on imaging elements using the NanoSIMS ion microscope at Lawrence Livermore)
Joey Miller (currently a medical student)
Wade Barranco (currently a Post-doc at the Cancer Center at Southwestern Medical School in Houston, TX)
Danny Kim (Post-doc, UCLA)

Grants and Clinical Trial

Support provided data for preparation of NIH RO1 and R21 grants submitted to the NIH

Support provided data for the design of a Clinical Trial to examine the chemoprotective effect of boron on men at risk for prostate cancer. This is current under review.

CONCLUSIONS

The project was designed to determine the relationship between elemental concentrations and the risk of prostate cancer. The work resulted in the identification of boron as a chemopreventative agent. This led to submission of a new grant for further study of the role of boron in cancer to the NIH-NCI. The project also provided data to support a clinical trial to determine if boron can reduce PSA levels in men at risk for prostate cancer. This proposal is currently under review for approval by the UCLA IRB. The work also resulted in the development of techniques to image the intracellular location of elements in prostate cells. This has been the most difficult part of the project and there remains a need for further work in this area.

References

1. Chan, JM, Stampfer MJ, Ma J, Gann, PH, Gaziano JM, Giovannucci EL. 2001. Dairy products, calcium, and prostate cancer risk in the Physicians' Health Study. Am. J. Clin Nut. 74:549-554.
2. Clark LC, Combs F, Turnbull BW, Slate EH, Chalker DK, et al. 1996. Effects of selenium supplementation for cancer prevention in patients with carcinoma of the

skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group. JAMA 276:1957-63.
3. Coffey DS. New insights and methodologies are needed to solve the many epidemiologic enigmas of prostate cancer. 2001. Epidemiologic Reviews 23, 1)
4. Feustel A, Wennrich R, Steiniger D, Klauss P. 1982. Zinc and cadmium concentrations in prostatic carcinoma of different histological grading in comparison to normal prostate tissue and adenofibromyomatosis (BPH) Uro Res 10:301-303.
5. Feustel A, Wennrich R. 1984 Zinc and cadmium in cell fractions of prostatic cancer tissues of different histological grading in comparison to BPH and normal prostate. Urol Res 12:147-50.
6. Greenwald P. 2001. Clinical trials of breast and prostate cancer prevention. J. Nutr.131:176S-178S.
8. Hayes RB. 2001. Gene-Environmental interrelations in prostate cancer. Epidemiologic Rev. 23: 163-172.
9. Kantoff PW, Febbo PG, Giovannucci E, Krithivas K, Dahl DM, et al. 1997. Cancer Epidemio. Biomarkers Prev. 6, 189-92.
10. Lindegaard PM, Hansen SO, Christensen JEJ, Andersen BB, Andersen O. 1989. The distribution of cadmium with the human prostate. Bio. Trace Ele Res 25:97-104.
11. Oldererеid NB, Thomassen Y, Purvis K. 1998. Selenium in human male reproductive organs. Human Repro. 13:2172-76.
12. Tvedt KE, Kopstad G, Haugen OA Halgunset J. 1987. Subcellular concentrations of calcium, zinc, and magnesium in benign nodular hyperplasia of the human prostate: X-ray microanalysis of freeze-dried cryosections. Cancer Res 47:323-328.
13. Zhang Z-F, Winton MI, Rainey C Eckhert CD. 2001. Boron is associated with decreased risk of human prostate cancer. FASEB J 15:A834.4.
14. Eckhert, C.D. Concentration and variation of boron, selenium and elements associated with cancer risk in non-tumor human prostate tissue. *FASEB J.* 2004; 18:A351.3 (351.3).
15. Cui Y. Winton M.I., Zhang, Z.F, Rainey C., Marshall J., deKernion J. B., Eckhert, C.D. Dietary Boron Intake and Reduced Risk of Prostate Cancer. *Oncology Reports* 11:887-892, 2004. Barranco W.T. and Eckhert C.D. Boric acid inhibits human prostate cancer cell proliferation. *Cancer Letters* 216:21-29, 2004.
16. Kim D. H. S., Faull K. F., Norris A. J., Eckhert C. D. Borate-nucleotide complex formation depends on charge and phosphorylation state. *J. Mass Spectrometry* 39:743-751, 2004.
17. Barranco WT and Eckhert CD. Inhibition of DU-145 Prostate Cancer Cell Proliferation by Boron and Selenium is Additive. *FASEB J.* 2005.
18. Barranco, W.T. Eckhert, C.D. Boric acid acts as a cADPR / RyR antagonist during inhibition of human prostate cancer cell proliferation. *FASEB J.* 2004; 18:A351.2 (352.2).
17. Kim, D.H, Faull, K.F., Eckhert, C.D. Determination of borate complex with cyclic ADP-ribose (cADPR) by electrospray ionization mass spectrometry (ESI-MS) *FASEB J.* 2004; 18: A351.4 (351.4).
18. Kim DH, Que Hee S, Norris A, Faull KF and Eckhert CD. Boric acid inhibit ADP-ribosyl cyclase non-competitively. *J. Chromatography A.* March, 2006.

19. Barranco WT and Eckhert CD. Cellular changes in boric acid-treated DU-145 prostate cancer cells. *Brit J. Cancer* 94:884-890, 2006.
20. Henderson K and Eckhert CD. Boric acid induces ER stress in DU-145 and LNCAP prostate cancer cell lines. *Soc. Tox*. 2006.

Appendices (published journal articles)

1. Barranco W.T. and Eckhert C.D. Boric acid inhibits human prostate cancer cell proliferation. *Cancer Letters* 216:21-29, 2004.
2. Kim D. H. S., Faull K. F., Norris A. J., Eckhert C. D. Borate-nucleotide complex formation depends on charge and phosphorylation state. *J. Mass Spectrometry* 39:743-751, 2004.
3. Barranco WT and Eckhert CD. Cellular changes in boric acid-treated DU-145 prostate cancer cells. *Brit J. Cancer* 94:884-890, 2006.Kim DH, Que Hee S, Norris A, Faull KF and Eckhert CD. Boric acid inhibits ADP-ribosyl cyclase non-competitively. *J. Chromatography A.* 1115:246-252, 2006.
4. Barranco WT, Hudak PF and Eckhert CD. Evaluation of ecological and in vitro effects of boron on prostate cancer risk. *Cancer Causes Control* 18:71-77, 2007.
5. Barranco WT, Hudak PF and Eckhert CD. Erratum to: Evaluation of ecological and in vitro effects of boron on prostate cancer risk. *Cancer Causes Control* 18:71-77, 2007, Published Online: http://dx.doi.org/10.1007/s10552-007-0077-8, *Cancer Causes Control*, March, 2007.

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Boric acid inhibits human prostate cancer cell proliferation

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Abstract

The role of boron in biology includes coordinated regulation of gene expression in mixed bacterial populations and the growth and proliferation of higher plants and lower animals. Here we report that boric acid, the dominant form of boron in plasma, inhibits the proliferation of prostate cancer cell lines, DU-145 and LNCaP, in a dose-dependent manner. Non-tumorigenic prostate cell lines, PWR-1E and RWPE-1, and the cancer line PC-3 were also inhibited, but required concentrations higher than observed human blood levels. Studies using DU-145 cells showed that boric acid induced a cell death-independent proliferative inhibition, with little effect on cell cycle stage distribution and mitochondrial function.

Keywords: Boron; LNCaP; DU-145; PC-3; Necrosis; Prostate cancer

1. Introduction

Boron has been shown to be beneficial for many species, but its cellular processing in animals remains obscure. The boron atom has a high affinity for oxygen and in nature is present in the form of borates [1]. Soluble forms include boric acid $B(OH)_3$ and the monovalent anion $B(OH)_4^-$, with the presence of the dominant form dependent upon solvent pH. In plasma, boric acid predominates and its concentration reflects dietary intake and respiratory exposure [2].

Boron is known to be important for animal cell replication and development, but the underlying mechanisms remain obscure. Boric acid stimulates embryonic growth in trout [3] and is essential during the pre-blastula cleavage stage of zebrafish [4]. In frogs (*Xenopus*), boron deficiencies interfere with normal oocyte maturation, embryonic growth and morphogenesis [5]. Deficiencies in *Xenopus* also lead to inhibition of oocyte germinal vesicle breakdown, possibly due to an alteration in progesterone receptor binding [6].

Evidence leading to the hypothesis that boric acid may be anti-carcinogenic was derived from epidemiological screening, where the risk of prostate cancer was observed to be inversely proportional to dietary intake of boron in a dose responsive manner [7,8]. Boric acid has also been reported to inhibit the growth of LNCaP prostate tumors in nude mice [9]. In the present paper, we report that boric acid

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JMS

Borate–nucleotide complex formation depends on charge and phosphorylation state

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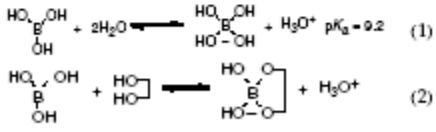
Flow injection analysis with electrospray ionization mass spectrometry was used to investigate borate–nucleotide complex formation. Solutions containing 100 μM nucleotide and 500 μM boric acid in water–acetonitrile–triethylamine (50 : 50 : 0.2, v/v/v; pH 10.3) showed that borate complexation with nicotinamide nucleotides was significantly influenced by the charge on the nicotinamide group and the number of phosphate groups on the adenine ribose. Borate binding decreased in the order of NAD^+, NADH, $NADP^+$ and NADPH. To investigate the relationship between complex formation and phosphorylation, association constants (K_A) of borate–adenine (AMP, ADP, ATP), –guanine (GMP, GDP, GTP), –cytidine (CMP, CDP, CTP) and –uridine (UMP, UDP, UTP) complexes were compared. The results showed that the number of nucleotide phosphate groups was inversely proportional to the relative abundance of the borate complexes, with the K_A of borate–nucleotide complex decreasing in the order mono-, di- and tri-phosphates (AMP ≈ GMP ≈ CMP ≈ UMP > ADP ≈ GDP ≈ CDP ≈ UDP > GTP > ATP ≈ CTP ≈ UTP). At pH 7.4, using ammonium bicarbonate buffer, only borate–NAD^+ complex was observed. This indicates that the borate–NAD^+ complex may be the most physiologically relevant of those studied. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: borate–nucleotide complexation; nucleotide; association constant; dissociation constant

INTRODUCTION

In aqueous solution, boron exists as either boric acid ($B(OH)_3$) or borate ($B(OH)_4^-$) ions, as shown in Eqn (1). Since the pK_a of boric acid is 9.2, at intracellular pH, nearly all free boron exists as boric acid. Boric acid reacts with polyhydroxy compounds, leading to a decrease in pH (Eqn (2)).[1–4] Complex formation between boric acid and polyhydroxy compounds depends on the pH of the solution and the structure of the polyhydroxy compounds, and we have previously shown that this is true for boric acid and NAD^+.[5] Van den Berg *et al* showed that in the case of diols, *cis*-1,2- are favored over *trans*- or 1,3-diols, and five-membered ring are preferred over six-membered ring 1,2-diols.[6] Therefore, a ribose ring with 2,3-*cis*-diol may be an important reactive site for interaction with boric acid.



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Boron is ubiquitous in the environment and is present in all living organisms. In 1923, boron was shown to be an essential nutrient for plants,[7] but it was not until 1996 that it was discovered to be structural component of the cell wall.[8,9] Recently a transport mechanism for boron in plant roots was identified that has low homology to certain kidney transport proteins.[10,11] Boron has also been shown to be a component of autoinducer (AI-2), a signaling compound regulating colony-wide gene expressions in Gram-positive and -negative bacteria.[12] Boron was recently shown to promote embryonic growth[13] and to be essential for post-fertilization cell growth in fish[14] and frogs.[15] Human studies suggest that boron may be important for bone strength,[16] cognitive performance[17] and serum 17β-estradiol levels.[18] Dietary boron intake has also been reported to reduce the risk of prostate cancer.[19] However, toxic effects of excess boron have also been reported. At high concentrations of oral intake, boric acid caused degeneration of seminiferous epithelium of rodents.[20] Although the mechanisms for these effects remain obscure, the long-known complexation of boric acid to the *cis*-diol moiety of ribose sugars suggests that a similar reaction may underlie its biological effects in animals. Since nucleotides are among the most abundant and metabolically important molecules containing the ribose *cis*-diol moiety, a ranking of borate–nucleotide complex by association constants could provide insight for understanding the physiological effects of borate.

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Cellular changes in boric acid-treated DU-145 prostate cancer cells

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Epidemiological, animal, and cell culture studies have identified boron as a chemopreventative agent in prostate cancer. The present objective was to identify boron-induced changes in the DU-145 human prostate cancer cell line. We show that prolonged exposure to pharmacologically-relevant levels of boric acid, the naturally occurring form of boron circulating in human plasma, induces the following morphological changes in cells: increases in granularity and intracellular vesicle content, enhanced cell spreading and decreased cell volume. Documented increases in β-galactosidase activity suggest that boric acid induces conversion to a senescent-like cellular phenotype. Boric acid also causes a dose-dependent reduction in cyclins A–E, as well as MAPK proteins, suggesting their contribution to proliferative inhibition. Furthermore, treated cells display reduced adhesion, migration and invasion potential, along with F-actin changes indicative of reduced metastatic potential. Finally, the observation of media acidosis in treated cells correlated with an accumulation of lysosome-associated membrane protein type 2 (LAMP-2)-negative acidic compartments. The challenge of future studies will be to identify the underlying mechanism responsible for the observed cellular responses to this natural blood constituent.

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Keywords: boric acid; prostate cancer; DU-145; migration; senescence; acidosis

Molecular Diagnostics

The element boron is nearly completely absorbed from drinking water and plant-derived foods in the gastrointestinal tract, and circulates in blood as boric acid (BA) (Price *et al*, 1997). Cells were once thought incapable of processing the element, yet this has since been disproved. Boron is utilised by bacteria in the structure of several antibiotics and autoinducer-2, a signalling molecule utilised during interspecies quorum sensing (Chen *et al*, 2002; Semmelhack *et al*, 2004). Plants require the element for growth, flowering and seed formation, and obtain boron from soil pore water using a borate transporter, BOR1, expressed in root pericycle cells (Takano *et al*, 2002). A human homologue, the electrogenic, voltage-regulated, Na^+-coupled borate transporter NaBC1, was recently identified in human kidney tubular cells and may function to maintain plasma BA levels (Park *et al*, 2004).

There are several reports supporting boron as a chemopreventative agent against prostate cancer. An epidemiological study using data from the NHANES III database reported that the risk of prostate cancer in US men is inversely proportional to dietary intake of boron (Cui *et al*, 2004). The biological plausibility of this observation has been supported by cell culture and animal studies. Treatment of nude mice, injected with androgen-sensitive LNCaP prostate cancer cells, with BA caused a reduction in tumour growth of 25–38%, along with a reduction in plasma PSA levels of 88% (Gallardo-Williams *et al*, 2004). BA inhibits the activity of serine proteases, including prostate-specific antigen (PSA), presumably by binding to its active site (Bone *et al*, 1987; Gallardo-Williams *et al*, 2003). In culture, BA has been shown to inhibit the proliferation of LNCaP and the androgen-independent prostate cancer cell lines DU-145 and PC-3, in a dose-dependent manner (Barranco and Eckhert, 2004). Since DU-145 cells do not synthesise PSA, BA's mode of inhibiting proliferation is likely not to occur by inhibiting the conversion of IGFBP-3 to IGF-1, as proposed in LNCaP tumours (Gallardo-Williams *et al*, 2004; Sobel and Sadar, 2005). The present investigation was initiated to define morphological and molecular responses of DU-145 prostate cancer cells to BA, which might lead to an explanation of its antiproliferative properties.

In the current report, we examined the effects of pharmacological concentrations of BA on cell morphology and molecular markers of proliferation, senescence, metastasis and motility. We show that prolonged exposure to BA causes DU-145 cells to develop a flattened, angular phenotype with numerous vesicles appearing in the cytoplasm. These changes occur coincident with a decrease in the expression of cyclin proteins, p21 and P-MEK1/2, as well as a reduction in cell motility and invasion capacity. Finally, increased β-galactosidase activity reflects a conversion of DU-145s to a senescence-like cell.

MATERIALS AND METHODS

Experimental culture

DU-145, LNCaP, and PC-3 PCa cells, donated by Dr Allan Pantuck, were cultured in RPMI 1640 media (Invitrogen, USA) supplemented with 10% FBS, penicillin/streptomysin (100 U ml^{-1}; 100 μg ml^{-1}), and L-glutamine (200 mM) (Gemini Bioproducts,

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Boric acid inhibits adenosine diphosphate-ribosyl cyclase non-competitively

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Abstract

Adenosine diphosphate-ribosyl cyclase (ADP-ribosyl cyclase) is a ubiquitous enzyme in eukaryotes that converts NAD^+ to cyclic-ADP-ribose (cADPR) and nicotinamide. A quantitative assay for cADPR was developed using capillary electrophoresis to separate NAD^+, cADPR, ADP-ribose, and ADP with UV detection (254 nm). Using this assay, the apparent K_m and V_{max} for *Aplysia* ADP-ribosyl cyclase were determined to be 1.24 ± 0.05 mM and 131.8 ± 2.0 μM/min, respectively. Boric acid inhibited ADP-ribosyl cyclase non-competitively with a K_i of 40.5 ± 0.5 mM. Boric acid binding to cADPR, determined by electrospray ionization mass spectrometry, was characterized by an apparent binding constant, K_A, of 655 ± 99 L/mol at pH 10.3.

Keywords: ADP-ribosyl cyclase; Boron; Capillary electrophoresis; cADPR; NAD; Mass spectrometry; Enzyme kinetics

1. Introduction

Adenosine diphosphate-ribosyl cyclase (ADP-ribosyl cyclase) is widely distributed in nature and is expressed in over 40 different species of protists, plants, and animals [1–5]. The enzyme cyclizes NAD^+ to produce cyclic-ADP-ribose (cADPR) with the release of nicotinamide (Fig. 1) [6]. cADPR acts as a second messenger that mobilizes Ca^{2+} from the endoplasmic reticulum via activation of ryanodine receptors [7]. Three homologs of the cyclase with 30% sequence identity have now been identified [8–12]. There is a soluble cyclase present in the sea hare *Aplysia*, the membrane-bound lymphocyte antigen CD38, and another antigen BST-1. Mammalian CD38 is a multiple function cell surface molecule possessing both cyclase activity that converts NAD^+ to cADPR, and hydrolase activity that converts cADPR to ADPR. At pH 4.5, CD38 also converts $NADP^+$ to nicotinic acid adenine dinucleotide phosphate (NAADP), another second messenger that also triggers Ca^{2+} release from intracellular stores. However, these stores are different from those affected by cADPR [13].

The soluble *Aplysia* cyclase has been recombinantly produced in yeast and crystallized [14]. This enzyme only catalyzes the synthesis of cADPR. Lee [8] has proposed a catalytic model for the CD38 type based on an active site that consists of a highly conserved sequence containing 10 cysteine residues and three other critical residues: glutamate 179, which is the catalytic residue and lies in the catalytic pocket, and two tryptophan residues. The model proposes that the two tryptophan residues bind and fold the linear NAD^+ molecule, while glutamate attack releases the nicotinamide moiety. The adenine ring then reacts with the terminal ribose to form cADPR [8]. According to this model, disruption of NAD^+ folding in the active site may slow the catalytic activity of the enzyme. The CD38 type has about 25% sequence identity with the *Aplysia* type. Boron as boric acid and borate binds to NAD^+ [15] and therefore may affect the activity of ADP-ribosyl cyclase.

Boric acid has affinity for diol-containing compounds such as carbohydrates, where its strong complexation is now being

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ORIGINAL PAPER

Evaluation of ecological and in vitro effects of boron on prostate cancer risk (United States)

Wade T. Barranco · Paul F. Hudak · Curtis D. Eckhert

Received: 30 August 2005 / Accepted: 24 August 2006

Abstract
Objective To determine: (1) the correlation of prostate cancer incidence and mortality with groundwater boron and selenium concentrations; and (2) the impact of boron on prostate cancer cell proliferation during co-treatment with alternative chemo-preventative agents, along with boron pre-treatment effects on cell sensitivity to ionizing radiation.
Methods For regression analysis, data on prostate cancer incidence and mortality were obtained from the Texas Cancer Registry, while groundwater boron and selenium concentrations were derived from the Texas Water Development Board. Cultured DU-145 prostate cancer cells were used to assess the impact of boric acid on cell proliferation when applied in combination with selenomethionine and genistein, or preceding radiation exposure.
Results Groundwater boron levels correlated with a decrease in prostate cancer incidence ($R = 0.6$) and mortality ($R = 0.6$) in state planning regions, whereas selenium did not ($R = 0.1$; $R = 0.2$). Growth inhibition was greater during combined treatments of boric acid and selenomethionine, or boric acid and genistein, versus singular treatments. 8-day boric acid pre-exposure enhanced the toxicity of ionizing radiation treatment, while dose-dependently decreasing the expression of anti-apoptotic protein Bcl-2.
Conclusions Increased groundwater boron concentrations, across the state of Texas, correlate with reduced risk of prostate cancer incidence and mortality. Also, boric acid improves the anti-proliferative effectiveness of chemo-preventative agents, selenomethionine and genistein, while enhancing ionizing radiation cell kill.

Keywords Boron · Selenium · Prostate cancer · Texas · Groundwater · Ionizing radiation · Bcl-2 · Genistein · Ecological

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Introduction

The development of public health strategies, for the prevention and control of prostate cancer, has been hindered by a gap in our understanding of factors responsible for the large geographical disparity in disease risk. It is estimated that there were 232,090 new U.S. cases in 2005, along with significant regional variability in incidence and mortality [1].

Environmental exposure, by way of dietary intake, is receiving much attention as a contributor to prostate cancer prevention [2]. Several natural, plant-derived products are currently in clinical prevention trials, including: (n–3) polyunsaturated fatty acids, flaxseed, vitamin E, selenomethionine (SeM), soy protein isolate, isoflavones, genistein, lycopene and low-fat diets [3–5]. The largest prostate cancer clinical trial, SELECT, will determine the effectiveness of SeM and

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ERRATUM

Evaluation of ecological and in vitro effects of boron on prostate cancer risk (United States)

Wade T. Barranco · Paul F. Hudak · Curtis D. Eckhert

Erratum to: Cancer Causes Control 18: 71–77
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Figure 1 (page 74) of this article was corrupted. The correct version and legend is given overleaf. The publishers apologise for this error.

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